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(54) Title: A METHOD FOR STABILISING BACTERIA BY CENTRIFUGING TO A NON-AQUEOUS PASTE

(57) Abstract

There is provided a method of stabilising bacteria present in an aqueous growth medium the method consisting in separating the bacteria from the growth medium by centrifuging to make a non-aqueous bacterial paste and storing the paste in a protective environment. The paste may be centrifuged into an impermeable sheath to make a sausage or may be resuspended in liquid and perhaps encapsulated in gelatin.

* See back of page

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Amethod for stabilising bacteria by centrifuging to a non-aqueous paste.

Once bacteria have completed their growth cycle in a particular medium or environment, they will either start dying or form a spore which can withstand long periods of starvation, dehydration, or extremes of temperatures. Since most types of bacteria cannot form environmentally resistant spores, their viability can be enhanced only by finding conditions or chemicals which prevent or at least decrease their rate of dying. Examples of chemicals which can sustain non-spore-forming bacteria from decomposition with time have been patented previously by Horsfall et al in 1976 using aqueous sulfide levels and by Wong et al in 1987 using aqueous azide levels. These inhibitory chemicals caused nonsporulating bacteria to remain viable over long periods of time without bacterial lysis, cell death from disintegration of the cell wall. In addition, recovery from long term storage was rapid from these aqueous suspensions of sulfide and azide. This is in contrast to the much slower recovery of viability from lyophilization, freeze-drying, or air drying of the bacteria either in the presence of some support media like bran or cereal or without any supplemental media at all.

These chemical systems for rendering bacteria dormant are simple to administer and provide an aqueous suspension ready to use and apply to the desired process. Their main disadvantage is that the levels of bacteria within these suspensions are low, and relatively large volumes of water are a part of the product components. This causes difficulty both in storage, as the products are voluminous and heavy, and in shipping or transporting, as the cost of shipping large volumes of water is high.

In addition, the process of sporulation, as

well as bacterial growth during the last stages of food or nutrient consumption, can involve the production of extracellular enzymes from the bacterial cytoplasm or from excretion. The purpose of these enzymes can be to create food for the bacteria from particulate organic matter. Since these enzymes can attack the components of the bacterial cell wall, their presence is deleterious to any but rapidly growing and reproducing bacteria. It would be a distinct advantage to be able to store bacteria grown to high levels and in the presence of these exoenzymes, free of their deleterious effects. Of course, removing totally the aqueous environment from the bacteria through freeze or air drying can accomplish this condition. This technique also concentrates all the salts as well as the enzymes and bacterial components of the system being dried. This may be one of the reasons for the high levels of bacterial death experienced during these drying techniques. The present invention seeks to provide an improvement.

According to the invention there is provided a method of stabilising bacteria present in an aqueous growth medium the method consisting in separating the bacteria from the growth medium by centrifuging to make a non-aqueous bacterial paste and storing the paste in a protective environment.

The protective environment may comprise an impermeable sheath made of Visking, Saran or like material. Alternatively, the protective environment may be liquid, preferably non-aqueous, but possibly aqueous and possibly water. Re-suspension of the paste in a liquid allows the bacteria to be very much more concentrated than in their original environment. Preferred non-aqueous liquids are glycerol, glycol or

the like.

The paste, either itself or when re-suspended in liquid, may be encapsulated in a non-permeable but dissolving or disintegrating degradable membrane such as gelatin.

One can remove the aqueous environment from the bacterial suspension without concentrating the existing level of any soluble component of the mixture by centrifuging the bacteria from their growth media. This will result in a bacteria-free solution and, more importantly, a bacterial paste almost totally free of water, salts and enzymes which would attack the bacterial cells and decrease viability considerably.

Because bacteria have a wet weight of about 10^{-12} grams per cell, the bacterial paste would contain 10^{12} cells per gram or 10^{15} cells per kilogram of paste. Since such a paste could provide for many volumes of bacterial suspension containing a bacterial concentration or level of one hundred million (10^8) cells per milliliter, for example, one kilogram of such a paste containing 10^{15} cells would make ten thousand (10^4) liters of such a suspension.Thus a paste weighing one kilogram (two and two tenths pounds) would substitute for a shipping container of the above aqueous suspension shipped as such. The cost differential would be several thousand dollars based upon sea freight rates. The air freight rate differential would be very considerable.

The preparation of such a paste is critical to retain bacterial viability and longevity. The centrifuged paste must be kept cool, free from evaporative losses and free from oxygen. To do this, it is necessary to use a continuous flow centrifuge such as a Sharples and an insert within the centrifuge made of a material like Visking or Saran so the

bacteria are deposited inside a cylindrical sheath as one would pack a sausage. The process described would produce a bacterial paste contained inside an impervious casing which would not age, since the aqueous environment, the enzymes, the nutrients, and the salts would be removed during the centrifugation process and remain with the water phase. Because the food for the bacteria remains in the aqueous concentrate, one could stop bacterial growth at any stage of its development, centrifuge them and retain the bacteria free of any environmental influence until resuspension of the bacteria back into an aqueous suspension which could be accomplished using a simple food blender like a commercially available Waring or Oster machine. Storage of the so-called bacterial sausages prepared by centrifugation could easily be accomplished in a commercial refrigerator and since the tubing would prevent dehydration (Saran being impervious to water), the refrigerator would not dehydrate the contents beyond the degree accomplished by the centrifuge.

In order to ulitize the bacterial mixture present in the sausage as a bacterial seed to grow a new culture, a section could be cut from the sausage and added to whatever growth medium one wished to use. Then bacterial growth could be initiated using methods any microbiologist would be competent to implement.

Such bacterial sausage preparations can be used for many purposes especially pertaining to but not limited to the wastewater treatment industry such as:

1) Preparation of mixed cultures for general wastewater or other type of treatment.
2) Maintaining cultures of bacteria from existing facilities that may from time to time be subject to toxic or conditional shock. These

stored cultures would provide a means of immediate on site relief from such eventualities by having immediately usable bacteria with which to grow a dense seed culture to be introduced into the plant.

3) Specialised preparations of mixtures of bacteria could be prepared using both the bacteria natural to the existing facility and those deemed to be needed to supplement the natural bacteria in that facility. Such a sausage could function to restart a treatment plant severally affected by conditions or toxic problems or as a supplemental seed to optimise and enhance operations of the plant on a regular and continuous basis.

4) The sausage provides a means to be able to transport huge numbers of viable bacteria in a concentrated and immediately available form from one site to another, even across vast distances at minimal expense.

Since the bacterial suspension in the sausage is free, for all intents and purposes, of food and water, the possibility of additional growth or death is virtually eliminated and a stable, yet immediately available, bacterial mixture is at hand.

Once a bacterial paste has been produced as described above, a highly concentrated bacterial mixture is available for use as a system to provide needed functions and capabilities to a facility. Such a system could be further enhanced by a method for rendering bacteria dormant and the subsequent products produced thereby in a non-aqueous media described below.

The capability to distribute bacteria in concentrated form into sediments at the bottom of

receiving bodies of water, treatment facilities, or aquaculture processes would provide a major advantage for the treatment and removal of those sediments. Systems now available rely primarily upon the physical injection of the bacteria into such systems as described above. If one could incorporate the bacteria prepared as a centrifuged paste into a liquid medium that was non-aqueous, denser than water, and yet readily soluble or miscible in water, the storage, distribution and utilisation of the bacteria would be enhanced considerably. There are several examples of such liquids available that would provide minimum effect on the environment and yet supply the needed prerequisite of a liquid system totally miscible with water. Some are:

glycerol - glycerin
ethylene glycol
1,2-propylene glycol
1,3-propylene glycol.

Introducing the bacteria from a paste into such media would result in a stable suspension because, even though these liquid media are biodegradable, there would be neither water nor nutrients available for bacterial growth in their preparation.

The growth of bacteria or, for that matter, any living form, requires four basic ingredients; carbon, nitrogen, phosphorus, and water. Since the bacteria have been centrifuged away from the nutrients used for their growth and most of the water in which they were growing, resuspending them in an anhydrous liquid, like glycerol, for example, will not provide the needed conditions for their growth, since neither nitrogen, phosphorus, nor water is part of glycerol, ethylene glycol, or the various isomers of propylene glycol. The bacterial suspensions which are formed

from the bacterial paste added to the non-aqueous, anhydrous liquids will be deficient in the nutrients necessary for bacterial growth and replication until they are added to a growth sustaining system. The stability of the system is provided by the growth limiting and anhydrous condition of the liquid suspension itself.

Since these liquids are totally miscible with water, the liquid suspension of bacteria produced with these materials would be immediately dispersed once introduced into an aqueous environment. Growth would ensue if the necessary nutrients like phosphorus and nitrogen were available. The original organic liquid used to create the suspension would then serve as a food source for the initiation of bacterial growth in the new aqueous environment until it was diluted beyond utility or consumed. Thus, once a growth sustaining environment is created for the bacteria, they will proceed to grow and reproduce once again, providing the cleaning functions of their metabolic capabilities. This technique holds the bacteria viably in a state of suspended animation until they are needed, fully capable of reactivation by the simplest procedure of direct introduction into a growth sustaining environment.

The characterists of the four liquid suspending chemicals chosen as examples herein are as follows:

Compound	density	m.p.	b.p.	solubility
Glycerol	1.269	20	290	infinite H_2O
Ethylene Glycol	1.1088	-11.5	199	infinite H_2O
1,2-Propylene Glycol	1.0361	-	189	infinite H_2O
1,3-Propylene Glycol	1.0597	-	213.5	infinite H_2O

These characteristics provide the suspension with several important capabilities.

1) It will not freeze at 0 degrees C, or even -20

degrees C.

2) It will not vaporise or boil at 100 degrees C.

3) It is not harmful to humans to handle and, in some cases, it may even be injested.

4) It is not inhibitory to bacterial growth at all.

5) It provides long term storage capability.

Once the bacterial suspensions described above have been created, liquid systems more dense than any known body of water on earth are on hand. There will be times when it will be advantageous to be able to introduce these bacterial suspensions into the organic muck layer at the bottom of a water body. To accomplish this a medium must exist to prevent the bacterial suspension from being dissolved by the water and just carried away with it. In fact it may be useful to be able to introduce these suspensions of bacteria into any system in which a slow release of the bacteria is deemed necessary. Without a suitable shell to prevent the dissolution of the suspending liquid, such a process is not possible. A method for incorporating bacteria suspended in a substance more dense than fresh or sea water into a form to provide a system for slow delivery is hereby described.

Encapsulating the liquid suspension produced using a material like gelatin which would allow a slow release of the bacterial suspension over time after the encapsulated liquid had sunk to the bottom of the water body would provide just the necessary system to allow for isolated use of the liquid suspension. Of course any solid or semi-solid coating that would allow a period of time to elapse before allowing the release of the liquid suspension contained within it would suffice. One could imagine a number of different coatings to accomplish this end. The basic

requirement for the coating would have to be limited solubility in water and insolubility in the liquid used to create the suspension of bacteria it coated. Since such systems are readily available commercially and in use in the manufacture of pills like vitamins they will not be discussed herein. In the case of glycerol, for example, a capsule similar to that sold as Vitamin E would be entirely sufficient.

CLAIMS

1. A method of stabilising bacteria present in an aqueous growth medium the method consisting in separating the bacteria from the growth medium by centrifuging to make a non-aqueous bacterial paste and storing the paste in a protective environment.

2. A method as claimed in claim 1 wherein the protective environment comprises an impermeable sheath.

3. A method as claimed in claim 1 wherein the sheath is made of the material Visking.

4. A method as claimed in claim 1 wherein the sheath is made of the material Saran.

5. A method as claimed in claim 1 wherein the protective environment is a liquid environment in which the paste is re-suspended.

6. A method as claimed in claim 5 wherein the liquid environment is aqueous.

7. A method as claimed in claim 6 wherein the liquid is water.

8. A method as claimed in claim 5 wherein the liquid environment is non-aqueous.

9. A method as claimed in claim 8 wherein the liquid is glycerol, glycol or the like.

10. A method as claimed in any of claims 5 to 9 wherein the liquid containing the paste is encapsulated in a non-permeable but dissolving or disintegrating degradable membrane.

11. A method as claimed in claim 10 wherein the membrane is gelatin or the like.

12. A bacterial preparation consisting of a non-aqueous bacterial paste derived by centrifuging contained within an impermeable sheath.

13. A bacterial preparation consisting of a non-

aqueous bacterial paste derived by centrifuging contain d in an non-aqueous liquid.

14. A bacterial preparation consisting of a non-aqueous bacterial paste derived by centrifuging re-suspended in water.

15. A bacterial preparation as claimed in claim 13 or claim 14 within a capsule.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01093

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 1/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	DE, C, 513511 (O.K. SAUER) 28 November 1930, see the whole document	1,5, 14
A	FR, A, 1272116 (M.L.H. HUBLOT ET AL) 9 August 1960, see the whole document	2-4,10-13
A	EP, A2, 0202409 (MILES LABORATORIES, INC.) 26 November 1986, see claim 6 and 7	11

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd October 1990	29 OCT 1990
International Searching Authority	**Signature of Authorized Officer**
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/01093

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-C- 513511	28/11/30	NONE	
FR-A- 1272116	09/08/60	NONE	
EP-A2- 0202409	26/11/86	JP-A- 61219380	29/09/86

For more details about this annex : see fficial Journal of the European patent ffice, No. 12/82

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